STUDIES ON THE LIFE-HISTORY OF PROTOZOA. III, THE SIX HUNDRED AND TWENTIETH GENERATION OF PARAMŒCIUM CAUDATUM.[1]

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Over 65 years ago, Ehrenberg came to the conclusion that the Protozoa are potentially immortal. Having all of the necessary organs and losing nothing in reproduction, there was no reason at all, he argued, why they can not live indefinitely. Shortly after this, Dujardin published a work in which he stated that Protozoa have periods of activity alternating with periods of rest, and Ehrenberg's view was vigorously contested.

Among contemporaneous biologists, the matter has been taken up, on the one hand, by Weismann and his followers, on the other by Maupas and those who see in Maupas' work the best weapon with which to combat Weismann. The matter at issue involves an interesting question, *viz.*, will protoplasm having every opportunity to exercise its various activities, and without being specialized through differentiation to the performance of limited functions, continue to live indefinitely, or will it gradually become exhausted and die as do the multicellular animals? Weismann, following the line of reasoning pointed out by Ehrenberg, maintains the former point of view, while Maupas, on the other hand, upholds the latter and argues, that, as the race becomes older, the vitality becomes more and more reduced, until finally, the Protozoön, like the Metazoön, dies from old age. Both writers have had to take into consideration the factor of conjugation with the accompanying phenomena which were almost entirely unknown to the earlier controversialists. With Weismann, conjugation is merely one of the vital functions of protoplasm, to be considered in the same light as feeding or excreting and not in any way interfering with the theory of immortality. Maupas

[1] A lecture delivered at the Marine Biological Laboratory, Woods Holl, Mass., July 28, 1902.

argues that, like Metazoa, the race would show unmistakable signs of senile degeneration and would die of old age were it not for conjugation which, in some unknown way, restores the vitality and ensures a new cycle of generations. According to Maupas, therefore, it would seem that the protoplasm of the unicellular organisms is not capable of the perpetual exercise of the ordinary vital functions. His conclusions lead to the assumption that, after conjugation, the individuals have a certain potential of vitality which is gradually used up in the course of a more or less definite number of generations, and which can be restored again and again by conjugation.

The general theories involved in this discussion seem to me important enough to warrant further experimental tests, and with this in mind, I started the culture of two different lines of *Paramœcium caudatum*, on the 1st of February, 1901, and these have been under continual observation ever since. One of the lines died out last month, in the 570th generation, the other is still living in the 665th generation.

One definite aim at the outset was to ascertain whether a given form under culture would pass through a more or less clearly-marked cycle of activity to die ultimately of old age as Maupas maintained. Another aim was to find out whether there is a well-marked degeneration of different parts of the cell as age advances. These aims merely called for the repetition of Maupas' work in the case of one organism and were interesting enough in themselves. There was still another aim, however, which lent added zest to the work and made every stage one of fascination. This aim was to find out, if possible, the significance of conjugation and why it is that vitality is renewed by this means. It seemed to me that if Professor Loeb could induce artificial fertilization in the echinoderm egg, it should be possible to induce artificial rejuvenescence in *Paramœcium*. The first definite problem that suggested itself was the discovery of some substitute for conjugation that could be used when the periods of physiological depression were imminent. The experiments have justified the assumption that such substitutes would prove successful, for I have been able to carry these minute organisms, not through merely one period of so-called degeneration, but

through five such periods, and have been able to rejuvenate them "parthenogenetically" five successive times. I feel that I am now in the position to confirm the early observation of Dujardin that, in *Paramœcium* at least, there are more or less regular periods of vigor and depression which alternate with each other in fairly regular succession. At each period of depression, the organisms, had they not been stimulated in some way, would have died, thus showing that they were physiologically worn out. The subtitle of this paper is taken from one of the periods of depression when the last survivors of a long line were nearly lost.

The methods employed in the investigation are practically the same as those used by Maupas. The infusoria are kept in cells in depression slides filled with a food medium of hay-infusion. The original *Paramœcium* of the A series was taken from the pond water of Van Cortland Park, New York, and transferred to one of these cells. In two days it had divided three times and of the resulting eight individuals four were isolated in the same way as the first had been, while the remainder were set aside as "stock." The four lines thus started have been maintained throughout and give a better basis for the study of vitality of the race than one line could possibly have given. A second series—B—was started at the same time with an individual *Paramœcium* from Fort Lee, New Jersey, and four lines of this series were likewise carried on with the A series. Individuals are isolated every one or two generations and the records are kept in the form of a table showing the number of generations since the last isolation and the total number since the beginning.

The division-rate is taken as the measure of vitality, for it represents the rate of metabolism, growth and reproduction. A better index of the general vitality could not be found and while fairly constant from day to day, its fluctuations mark out clearly the periods of vigor and depression. The variations of the rate are difficult to follow when the daily records alone are considered and the cultures had been under way for ten months before a method was discovered of representing it graphically. When the daily rate of division is plotted the fluctuations of the curve, due partly to varying temperature and the food conditions, are perplexing and it is extremely difficult to compare the con-

dition of the general vitality at different periods by this means. Good results were finally obtained by averaging the number of divisions per day for definite periods, and plotting the averages. By this method of illustration the condition of the general vitality at any period desired can be seen at a glance. (See Fig. 1.)

The division-rate, as represented by such a curve, continued without noticeable variations from the beginning of the experiments until the latter part of the following July. About the 20th of that month the organisms began to die off at an alarming rate, indicating that a period of depression had apparently set in, or "degeneration" in the Maupas sense. Now was the time, if at all, to experiment with substitutes for conjugation, and in conformity with my original plan, the first trials were made with a change of diet. The organisms had been living steadily on the bacteria that develop in hay-infusion (*Bacillus subtilis* mainly), and various substitutes were now tried. Vegetable infusions of different kinds gave no improvement but meat infusions proved successful. The first experiment with the latter was with teazed liver which was added to the usual hay-infusion. The result was very gratifying for the organisms began immediately to grow and to divide, the rate of division rising from 5 to 9 divisions in successive 10-day periods. The effect of the liver was not lasting, however, and in the following 10-day period, the rate fell off to one division in five days or two-tenths of a division per day. During this time the organisms had not been continuously on liver treatment but had been transferred to the usual hay-infusion diet after 48 hours in the liver medium. At the end of the first 10-day period in August the cultures were again dying off, this time more rapidly than before and the renewed use of teazed liver had absolutely no effect. One thing after another was now tried without success, the organisms meanwhile growing weaker and weaker. Finally they were transferred to the clear extract of lean beef made by boiling fine pieces of beef in tap water. The effect of this medium was interesting, for, although it restored the weakened vitality, there was no rapid increase in the rate of division as when first treated with the teazed liver. The infusoria were, however, now large and vigorous and did not die unless transferred from the beef medium to the usual hay infusion. When this was attempted,

they would become abnormally active and would finally die. The division-rate gradually increased during the month of August until, in the last ten days, they averaged six generations. Finally, in September, the attempts to get them back on the old diet of hay-infusion were successful, and then the division-rate went up at once to twelve times in ten days, and a month later, they were dividing at the rate of fifty times a month.

This experience showed that "rejuvenescence" in the Maupas sense, can be brought about without the aid of conjugation. Had it not been for the extract of beef, or some other equally beneficial substance, all of the organisms would have died, and, like Maupas,[1] Joukowsky[2] and Simpson,[3] I should have assumed that their possibilities of continued living had been exhausted and that they had died from "old age" in the 198th generation.

From November 1st to December 10th the curve shows that the division-rate and the general vitality gradually decreased, and by the middle of December, a similar experience to that of midsummer threatened the cultures. Profiting by that experience I took the precaution of giving some of the individuals beef-extract before they got too weak, while others were continued on the hay-infusion. All of the latter died within a few days. Those that had been fed with beef-extract for 48 hours continued to live when put back in the hay-infusion, and they continued through another cycle of almost three months without treatment again with the beef.

From the middle of December on, the cultures were continued under slightly different treatment. Two of the four lines of As (A–1 and A–2) and two of the four lines of Bs (B–1 and B–2), were treated once per week for 24 hours with beef extract. This treatment was continued until the middle of March, when they were given hay-infusion without further variation. The other lines of As and Bs, *viz.*, A–3, A–4, and B–3, and B–4, were treated in December with the beef-extract and then kept on hay-infusion without further change. By this treatment it was possible to compare the general vitality under conditions of a more or less

[1] Maupas, E., *Archives d. Zoöl. expér. e. gén.* (2), VI. and VII., 1888, 1889.

[2] Joukowsky, D., *Verh. nat. Med. Ver. Heidelberg*, 1898, Bd. XXVI.

[3] Simpson, J. Y., *Proc. Roy. Soc. Edinburgh*, 1901.

regular stimulus, with the general vitality under conditions of an initial stimulus. The history of these experiments is shown graphically in the diagram shown on page 198.

On the first of January, all were in fairly good condition and the division-rate was about the same for all. All showed a decline in the latter part of February, and the rate of division rose for all of them in March. At this time there was a sudden warm period in which the temperature for that ten-day period increased 3 degrees on the average and this apparently acted as a mild stimulus. From this time on, however, the division-rate of the hay-fed individuals began to fall, and as soon as this fact was noticed, the beef treatment of the others was stopped. The hay-fed individuals did not recover their vitality and their division-rate continued to fall until finally stock and all died, apparently worn out. The beef-fed individuals, on the other hand, continued their high rate of division throughout the period of decline of their sister-cells, and did not show signs of diminished vitality until the first period in June. At this time, however, the rate fell very rapidly and the depression was clearly apparent in the daily records (See Fig. 1). The organisms at this time had not had beef-extract since March and they were accordingly transferred to it now with perfect confidence on my part that it would again prove to be the "elixir of life" for the *Paramœcium*. *This time, however, it failed to give the looked-for result, and the animals continued to die at a rapid rate.* Their appearance indicated that their trouble was not the same as in the previous periods of depression. Hitherto at such times the endoplasm appeared black under the microscope from the large numbers of gastric vacuoles with their food contents unchanged. At this time, on the other hand, the endoplasm was clear and free from such food masses, but the appearance of the protoplasm was singularly granular and different from that of a healthy individual. All changes that had proved beneficial before were now tried. These included not only liver, various vegetable infusions and the like, but salts of various kinds. Nothing seemed suitable for the organisms and they died off at a faster rate than ever. *When the last of the B series, stock and all, died in the 570th generation (June 16), and the A series became reduced to only 6 individuals in the 620th*

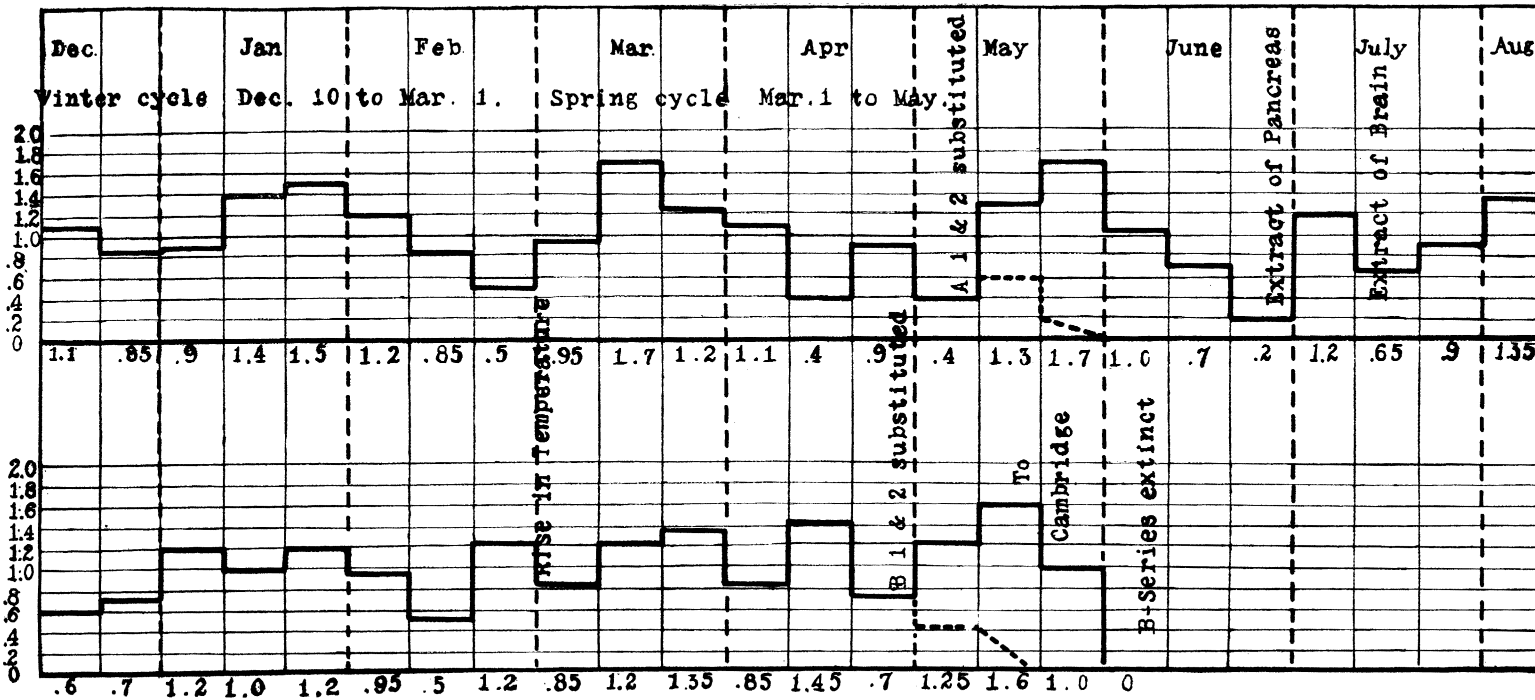

FIG. I. Curve showing the history of the cultures from December 10, 1901, to August 10, 1902. The upper curve represents the A-series, the lower one, the B-series. In the former, the daily division-rates for A–3 and A–4 are averaged and these again are averaged for 10-day periods. In the latter, B–3 and B–4 are averaged in the same way. A–3, A–4, B–3 and B–4 were given beef extract in December and were then continued on hay-infusion until all four lines became extinct in May. At this time it became necessary to substitute A–1, A–2, B–1 and B–2 for the weakening sister lines whose further history is indicated by the dotted lines. The ones that were substituted had been fed once per week for 24-hour periods with beef-extract, but this treatment was stopped in March and they were given hay-infusion from that time on. The cycles thus overlap, for the beef-fed lines in March were in the same stage, as regards stimulation, as the hay-fed ones were in December. The B-series became extinct in June; the A-series were saved by change of diet. Those that were "rejuvenated" by extract of brain were substituted for those that were treated with extract of pancreas as soon as it was seen that the latter did not have a permanent effect.

generation (June 27th) it looked as though the cultures were about to come to an end. The unusual appearance of the organisms made me think that the trouble was not with the digestion of food, but with its assimilation, and with this clue in mind I made extracts of sheep's brain and sheep's pancreas. Three of the six remaining individuals were put into the brain-extract and three into the extract of pancreas. The latter worked best and the 621st generation appeared the next day. The former, while giving slower results, produced healthy and normal organisms, and in three days there were more than 30 individuals, and enough to experiment with. After 48 hours' treatment with each of these extracts the organisms of the regular series were transferred to the usual hay-infusion. Since then the division-rate has been fairly constant, and, as stated at the beginning of this paper, they are to-day (July 28th) in the 665th generation.

The effect of the two extracts was very interesting. The individuals that were treated with pancreas had been in N/25 magnesium chloride for thirty minutes on the sixth day of May. Those that were treated with the brain-extract had been in N/1250 potassium phosphate for thirty minutes on the eighth of June. The division-rate of the former, notwithstanding the quicker start, soon fell behind that of the latter, and to-day the only ones alive are the descendants of those that had been fed with the brain-extract.

The interpretation of these facts involves the interpretation of many of the deepest problems of biology. If we could point out their significance, it would mean that we are in a position to throw light upon the ultimate secrets of vitality; secrets which have taxed the ingenuity of philosophers of all ages, and which lie at the bottom of modern biological research. At the present time the question cannot be answered, and it seems mere presumption to attempt an explanation. Nevertheless, there are sufficient data to warrant a tentative interpretation even though it is only a vague, shadowy impression of what may actually go on in living protoplasm.

The bacteria in the hay-infusion constitute the normal food of the Paramœcidæ. Of these, *Bacillis subtilis*, is, probably, the only one left alive after the infusion is raised to the boiling point, and

this organism, therefore, forms the staple article of diet for *Paramœcium* in culture. In the body of the infusorian the bacteria are collected in the usual gastric vacuoles where the first stages, at least, of digestion are carried on. No one has identified the proteolytic ferment which is active in this process. The work of Meissner, LeDantec, Fabre-Domergue, Greenwood and others has shown that something analogous to pepsin plays the most important rôle in the process, but nothing definite concerning the ferment is known. In an allied group, however, the Rhizopoda, some work has recently been done which may throw light upon the digestive processes of the Infusoria. H. Mouton,[1] working in Metschnikoff's laboratory, has succeeded in extracting the proteolytic ferment from a bacillus-eating *Amœba*. Tests of various kinds showed that the ferment is more like trypsin in its action than like pepsin, and Mouton comes to the conclusion that it is intermediate between the two. As the foods of this *Amœba* and of *Paramœcium* are probably similar in proteid composition, it is not improbable that the proteolytic ferments are much alike.

Whether or not the proteolytic ferment in *Paramœcium* is the same as that in *Amœba*, there is no doubt that some such active agent is operative in the digestive processes of our infusorian. Furthermore, there is a certain amount of evidence to indicate that it is this ferment-forming power that becomes weakened in the protoplasm of *Paramœcium* as the race grows older. Physiological "degeneration" is not due, as Maupas supposed, to the loss of some of the necessary organs of the cell through degeneration, nor is it due to inability to take food, for, when in the depressed condition, the *Paramœcium* still maintains a current of bacteria into the mouth-opening, and food vacuoles are still formed. Frequently when in this condition the organisms appear black because of the undigested food balls, a fact which indicates that it is the digestive function that wears out. In this connection it is interesting to note the effect of dilute alcohol on the general metabolism of *Paramœcium*. Mr. C. C. Lieb, work-

[1] H. Mouton. Recherches sur la digestion chez les Amibes et sur leur diastase intracellulaire. Thesis presented at the Faculté de sciences de Paris, 1902. Reprint pp. 1–60.

ing with *Paramœcium* from these cultures, found that alcohol will increase the division-rate above the control, by about 33 per cent. Under the action of the alcohol the organisms are always lively and appear transparent as though all food were rapidly digested. The result is interpreted as a stimulus to the digestive processes effected by the alcohol.[1] In the period of depression which is represented by the 620th generation, alcohol brought about no stimulus and the organisms died as rapidly as the ones not thus treated.

As the age of the race increases, it is conceivable that something in the chemical make-up of the protoplasm becomes depleted, something which cannot be replaced from the constituents of the ordinary food medium. With the change of diet, however, new substances are introduced into the protoplasm and renewed activity results. Thus with the change from hay-infusion to beef-extract, chemical substances are introduced into the protoplasm which could not be obtained from the hay-infusion. In the New York water there are certain chlorides, nitrates, etc., all in extremely minute quantities and these are present of course in the hay-infusion. They are also present in the beef-extract which is made with the same water, but in addition to these salts there are certain extractives from the beef such as potassium phosphate and chloride, lactic acid and lactates, etc., substances of quite a different character from those of the ordinary medium. Added to the fact of a different chemical medium is the fact of a difference in osmotic pressure, for the extract is more dense than the hay-infusion. The extract does not have a direct stimulating effect upon the digestive processes and upon division, for, while the organisms are immersed in it, there is a very slow division rate; when transferred again to the hay-infusion, however, they divide more rapidly than before. From this it appears that the extract is not a food in the ordinary sense but that it is a stimulant acting through the salts dissolved in it.

These results justify, I believe, the conclusion that, as the potential of vitality becomes reduced, something in the chemical

[1] Cf. Calkins and Lieb. Studies on the Life-History of Protozoa. II., The Effect of Stimuli on the Life-Cycle of *Paramœcium caudatum*. *Arch. f. Protistenk.* I., 3, 1902.

composition of *Paramœcium* wears out or disappears so that the ferment-forming activity is lessened. A change of diet restores the power. The restoration may be effected, not only by the extract of beef, but by clear chemicals as well. Thus potassium phosphate, potassium chloride, sodium and magnesium chlorides, have all given positive results in this direction, and it is probable that these substances contain the elements needed by the weakened protoplasm. We are justified, therefore, in regarding the artificial rejuvenation of *Paramœcium* as a phenomenon of the same order as the chemical fertilization of the egg.[1]

The general problem may be attacked from another point of view. What is it that conjugation does to renew the potential of vitality? The early conception of Butschli's that conjugation brings about a renewal of youth or a *Verjungung* of the participating organisms has been repeatedly confirmed by subsequent observers. A very significant fact came to light in the course of these experiments and one which seems to show that conjugation, like the action of the beef-extract, involves certain chemical phenomena. It will be recalled that Maupas maintained that endogamous conjugation is fruitless and the reason given was that, as the cultures grow older, the micronucleus degenerates and disappears. Maupas did not keep *Paramœcium* in culture for more than a few weeks, but the period was long enough for him to feel justified in assuming that this form is similar to the other forms examined by him. He declared that endogamous conjugations in this species (and he obtained many of them) are sterile because the micronucleus is lost through degeneration. I have preparations of this form made at intervals throughout the 665 generations, during endogamous and exogamous conjugation, at periods of depression and of vigor, and in none of them is the micronucleus absent. Upon this point Maupas was most assuredly mistaken.

From time to time As and Bs have been put together for the purpose of obtaining fertile conjugations. In the material set aside as "stock" conjugations have also frequently occurred, these of course, being endogamous. A comparison of "wild"

[1] Cf. Calkins. Studies on the Life-Cycle of Protozoa. I., The Life-Cycle of *Paramœcium caudatum*. *Arch. f. Entwickl.*, 1902.

conjugations with endogamous and exogamous unions is very instructive. By "wild" is meant pairs that are captured while conjugating in the natural pond water. There were only three pairs of these but of the six ex-conjugants five continued to live in the regular culture medium. Had the original number of pairs been larger, the result would be more satisfactory but I believe the proportion of fertile results would be as high as five out of six, or, expressed in percentages, as high as 83 per cent. Of the exogamous conjugations, *i.e.*, those of diverse ancestry, there were 24 pairs selected at different periods, and 48 ex-conjugants isolated, of which only three continued to live in the same culture medium, or about six per cent. Of the endogamous unions, *i.e.*, conjugations in which the gametes were of the same ancestry, 16 pairs in all were selected and 32 ex-conjugants isolated, of which two only continued to live, again about six per cent. How are we to account for the great discrepancy between the proportion of fertile wild ex-conjugants and those obtained from the cultures? *I believe that it can be accounted for on the ground of chemical differences in the composition of the protoplasm of the different individuals.* In the natural habitat of these forms no two individuals have the same environment for long periods and it may well be that no two parts of the pond have an identical composition. When two organisms unite, as *Paramœcium* unites, and part of the micronucleus of one unites with a similarly-derived part of the micronucleus of the other, the result is the formation of a new chemical substance in each organism, for the fusion nucleus in all probability is different in minute composition from either of the old ones. Now, on the other hand, when the organisms had been in the same culture medium for long periods, as was the case with the As and Bs of these experiments, there was no chance for them to be different from one another in chemical composition, and the ex-conjugants, after a few generations, died in the great majority of cases. In all such cases the conjugations were normal, micronuclei were exchanged, the old macronuclei became disintegrated and new ones were properly formed, but something was lacking and the ex-conjugants died. I believe this "something" has to do with the chemical make-up of nucleus and cytoplasm; and I further believe that the failure

which Maupas experienced in getting fertile endogamous ex-conjugants can be explained upon the same grounds. In one instance I treated an endogamous ex-conjugant with beef extract. The effect was most remarkable for it not only continued to live, *but it is still living in the 350th generation.* When it is remembered that the usual life-cycle of *Paramœcium* in culture is from 150 to 170 generations, this result is extremely interesting.

The facts of conjugation thus seem to fit in with the interpretation of the other results, to indicate that the protoplasm of this one-celled organism, like a galvanic battery, starts with a high potential of activity, which gradually fails with use, but which may be restored again and again by the proper chemical means. It is to such an assumption that we may turn for an explanation of the periods of depression that have occurred from time to time. The June period, at the 620th generation, appears to have been different from the earlier ones, for at this time, neither beef-extract, nor any of the salts that had been successful before, would restore the lagging energies. It was only the use of something entirely different from everything previously employed that finally saved the culture. It may have been the lecithine and allied substances in the brain extract, and it may have been the nucleinic acid in the pancreas extract; whatever it was, it apparently supplied something in the protoplasm of *Paramœcium* that had not given out before, but had failed at this particular time. Further, and more carefully-guarded experiments, must now be undertaken in the hope of finding out what these failures mean.

Turning again to the controversy regarding "old age" in Protozoa, it seems that we shall have to side with Weismann in the contention that these organisms have the potential of endless existence, and this without conjugation. The five times that the cultures of *Paramœcium* have been rescued at periods of depression, justify the belief that they may be saved as many times more, provided the time and patience required for such work are not out of all proportion to the results gained. It seems to be merely a matter of finding the right food, *i. e.*, the right chemicals. In nature, it is not improbable that the periods of depression, which are experienced in the laboratory, would be

absent, and that the division-rate would maintain a fairly uniform level. The chemical composition of a pond is constantly changing; every rainfall washes new salts into the water; every wind assists in turning the water over, and with these changes, it is probable that the organisms get new supplies of the substances necessary for their continued activities. When other things fail, the organisms may resort to conjugation, but we may consistently doubt whether this method of recuperation for Protozoa is as widespread in nature as hitherto supposed.

One other analogy is suggested by these experiments, *viz.*, the similarity of these periods of depression, and the subsequent proliferation, to tumorous growths of various kinds in the higher animals. The race of *Paramœcium* may be compared with the tissues composing the body of a Metazoön. When the cells composing these tissues approach the age-limit of the race, they divide less and less often; finally they come to a halt, so far as division is concerned, and senile degeneration begins, which ends in death. If, in a given tissue, after it has passed the period of active division, some stimulant be given at a local area, thus inducing a renewal of the dividing energy and proliferation of the cells of that area, the result, from a pathological point of view, would be a tumor; from a biological point of view, it might well be the rejuvenescence of the cells, as in the case of *Paramœcium*. As in the latter case, a number of agents might be responsible for calling out the increased activity, as, for example, a blow, a parasite, an enzyme, a changed condition of the immediate environment, etc. Any one of these might "rejuvenate" the tissue-cells in the same way that a chemical induces a new cycle of generations of *Paramœcium*.

MARINE BIOLOGICAL LABORATORY,
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